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Abstract

The optimal preservation of many Egyptian archaeological sites provides unique opportunities in the research into the evolution of synanthropic species, wild animals or plants, which benefit from close association with man-made human habitats. In this study we extracted and analysed ancient mitochondrial DNA (mtDNA) from three synanthropic insect species, two storage pests, *Sitophilus granarius* (N=8) and *Trogoderma granarium* (N=14), and the house fly *Musca domestica* (N=14), from Roman Qasr Ibrim, an Egyptian frontier site located in lower Nubia. The impact of different experimental variables on ancient DNA recovery was also evaluated, confirming that it is possible to extract endogenous ancient DNA from desiccated specimens while preserving the insect exoskeleton. A phylogenetic comparison of the Qasr Ibrim housefly mtDNA-COI (COI) with modern housefly sequences, revealed that they were genetically similar to modern Egyptian, Near Eastern, Indian, Japanese, and US/Canadian populations. As the now cosmopolitan houseflies were transported by human populations alongside domestic animals and crops and may have aided the spread of disease, these findings provide important information for these processes. While limited by the resolution of the comparative databases, our research suggests the existence of biological invasions and links across the Red Sea from Egypt to the Arabian Peninsula, and exchanges between India and Egypt. We demonstrate the great potential of fossil insect aDNA for reconstructing biogeographic and diachronic species distribution and for better understanding past environments.

Keywords: Fossil insects, aDNA, Roman Egypt, genetic diversity, biogeography

Highlights:

- Ancient mitochondrial DNA successfully extracted from nine *Musca domestica* specimens from the Roman levels of Qasr Ibrim, an Egyptian frontier archaeological site
- Comparison of extraction protocols confirms that is possible to extract DNA from ancient Diptera without morphological damage to the specimen
- Comparative analysis revealed affinities between Qasr Ibrim and modern *M. domestica* populations from Egypt, Israel, Saudi Arabia, India, that could be explained through ancient trade routes between these countries and also Japan and US/Canada which could be a result of more recent trade and the spread of houseflies globally
- The study of synanthropic insect species can inform about human movement, trade and past environments

1. Introduction

Ancient DNA research has recently made breakthroughs with new results relevant to biogeography, domestication, origins and spread of farming and disease (Hagelberg et al., 2015). Although there has been much research on human mobility and the dispersal of crops and domestic animals with humans, there has been little research on insects. Dispersal of synanthropic insects, which benefit from close association with man-made habitats, is strongly linked with movement of humans, the spread of farming, urbanisation and trade (e.g. Panagiotakopulu et al., 2010; Panagiotakopulu and Buckland, 2017). During the Holocene, biological invasions of many insect species from their primary natural niches into humanly defined, synanthropic environments have taken place. Palaearctic fossil records demonstrate their importance as indicators of human impact, including the spread of pathogens (McMichael, 2004; Panagiotakopulu and Buckland, 2018; Simberloff et al., 2013). While insect remains from archaeological sites have been used to investigate biogeography in relation to past synanthropic environments and ecosystems (e.g. Panagiotakopulu and Buckland, 2017), sequencing and analysis of insect ancient DNA (aDNA) remains relatively unexplored. As many insects are closely linked to the movement of human populations and their living conditions, insect aDNA has the potential to reveal new information both about the species involved and also about past human movement.

The recovery of insect aDNA has generally concerned specimens <150 years old and has been primarily used for cataloguing (Prosser et al., 2016). Sporadic attempts to sequence the DNA of fossil insects have yielded some success, primarily using museum specimens (Cotoras et al.,

2017; Heintzman et al., 2014). Insect DNA has been obtained from waterlogged samples recovered from Roman and medieval assemblages (King et al., 2009**)** and studies concerning Quaternary assemblages, focussing primarily on the ability to obtain amplifiable DNA and basic species identification, have also had some success (King et al., 2009**;** Thomsen et al., 2009**)**. Further research initiated by Reiss (Reiss et al., 1999; Reiss 2006) has pointed out problems with these early attempts concerning collection and curation procedures, the small size of insect specimens and finally the need to sacrifice unique specimens as part of the analysis.

Fossil insect research from Pharaonic and Roman sites has produced interesting results (e.g. Panagiotakopulu et al., 2010; Panagiotakopulu and Buckland, 2009; Panagiotakopulu and van der Veen, 1997), but there has been little aDNA research so far, partly as a result of permit issues from these well-preserved assemblages and partly due to the limitations of appropriate methodologies for aDNA extraction. Recovery and analysis of aDNA can be used in Egypt in multitude of ways: to reconstruct paleoenvironments, to analyse climate patterns or to develop an understanding of living conditions and to retrace ancient trade routes.

By adapting previously used extraction and genotyping techniques (e.g. Gilbert et al., 2007b) this study demonstrates the feasibility of extracting aDNA from insects of significant age and highlights the potential of the genetic information obtained for examining human palaeoenvironments and past ecological changes.

2. Materials and Methods

2.1. The site

Qasr Ibrim was a major settlement located in lower Nubia (Fig. 1), a region controlled intermittently by Egypt beginning as early as the 13th century BC and continuing to the early 19th century (Rowley-Conwy, 1988). During its Roman occupation, from c. 25 BC to 100 AD (Clapham and Rowley-Conwy 2010) (Table 1), the site was significant for the defence of the southern frontier of the Roman Empire in Africa. Regardless of the ultimate controlling power, Qasr Ibrim's location close to the Nile ensured that occupation, with perhaps one brief break, was mostly continuous until its final abandonment (Table 1).

Figure 1. Location map of Qasr Ibrim, including other archaeological sites mentioned in the paper.

Excavations have revealed preservation of organic materials with the remains of crop storage and processing, as well as domestic animals (Clapham and Rowley-Conwy, 2006, 2007). The excellent preservation of organic materials, from wood and basketry to plants and skins by desiccation (e.g. Driskell et al., l989; Rose and Edwards, 1998; Rowley-Conwy, 1991) allowed the recovery of aDNA from plant remains (Deakin et al., 1998; Smith et al., 2019). Results have also shown the local transition from six to two row barley at the site (see Palmer et al., 2009), provided the first evidence for cotton domestication in Africa (Palmer et al., 2012) and the earliest evidence of the RNA pathogen Barley Stripe Mosaic Virus (Smith et al., 2014).

Period	**Date**
Napatan	9th Cent BC–*c.* 4th Cent BC
Roman	*c.*25BC–AD100
Meroitic	100–350 AD
Post-Meroitic	350–550 AD
Early Christian	550–850 AD
Classic Christian	850–1100 AD
Late Christian	1100–1400 AD
Islamic	1400–1812 AD

Table 1. Chronology of occupation of Qasr Ibrim (after Clapham and Rowley-Conwy 2010).

Insect assemblages from the site include various species of beetle associated with extensive infestations of stored crops. Some of the most abundant insect species found at Qasr Ibrim samples are the granary weevil, *Sitophilus granarius* L., with several complete individuals recovered and the khaphra beetle, *Trogoderma granarium* Everts, with considerable numbers of complete specimens from deposits of the Late Christian/Early Islamic period. A large number of the puparia of house flies, *Musca domestica* L. were recovered from Late Napatan to Roman period deposits. *S. granarius* is flightless, with probable origins in the Fertile Crescent in rodent food caches and has a fossil record of expansion with the beginnings of agriculture (Panagiotakopulu and Buckland, 2018); the earliest record goes back to 7th millennium BC at Atlit Yam on the Levantine coast (Kislev et al., 2004). *T. granarium* is another cosmopolitan pest of grain and can be found on a range of other products (Fogliaza and Pagani, 1993; Peacock, 1993) with suggested origins in India (Banks, 1977). The earliest fossil records of the species are from New Kingdom el-Amarna, in middle Egypt (Panagiotakopulu, unpubl.). *Musca domestica,* the housefly*,* is thought to be endemic in the Nile valley; from there it spread around the Old World, with records northwards to Neolithic Alvastra in southern Sweden by the Neolithic (Skidmore in Lemdahl, 1995).

2.2. Laboratory Analysis

A total number of 36 desiccated specimens from 3 species: *Musca domestica* (14 samples) *Trogoderma granarium* (14 samples), and *Sitophilus granarius* (8 samples) were taken from the Roman deposits at Qasr Ibrim. These insect specimens were recovered from sediment samples obtained for palaeoecological analysis, dry-sieved through a 250 mm sieve and the residue sorted under a stereomicroscope. Specimens were identified using entomological keys, the Osborne collection and additional specimens at the School of Geosciences, University of Edinburgh. Complete specimens of individuals were selected where possible.

As part of this research samples of seeds and insects were sent for radiocarbon dating in order to confirm and refine the archaeological chronology. Although the intention was to use the methodology for dating of insect chitin (Panagiotakopulu et al., 2015; Tripp and Higham 2011) the samples were very small and this pre-treatment could not be used. However the dates obtained from seeds from the same deposits essentially overlapped with the insect samples and archaeological dating, indicating that desiccated insect samples do not involve the methodological issues which occur with some waterlogged fossil insect material or specimens stored in ethanol. The three samples selected for AMS dating and results are presented in Table 2.

Lab code	Sample no.	Material	Radiocarbon age (14C yr BP ±1σ)	Cal. AD range (±2σ)	δ13C (‰)	C/N
OxA-37677	QI-84-102	*Triticum cf aestivum* L.	379±24	1446-1630 AD	-25	
OxA-37791	QI-84-102	*Trogoderma granarium* Everts	351±24	1457- 1635 AD	-23	5
OxA-37793	QI-86-31 4	*Musca domestica* L.	1987±27	45 BC-68 AD	-22	6

Table 2. Radiocarbon dates from insect and seed samples analysed to confirm and refine the archaeological chronology. Calibration was performed using IntCal13 (Reimer et al., 2013) and the software OxCal v4.3.2 (Bronk Ramsey, 2017).

For aDNA analysis, precautions were taken in order to limit exposure of the specimens to sources of contamination. For all DNA extractions and PCR preparations a dedicated ancient DNA laboratory with positive pressure and UV lights was used, followed by PCR amplification in a separate modern DNA laboratory. Full protective clothing, including a suit, gloves, face mask and goggles were worn in the aDNA laboratories. The aDNA laboratory was cleaned after every use with bleach and sterilised under UV lights overnight.

DNA was extracted using the digestion buffers described in Gilbert et al., 2007b and Thomsen et al., 2009. Prior to immersion in the digestion buffer, samples were placed in a UV cross-linker for 10 minutes (5 minutes on each side of the insect) in order to remove surface contamination. Samples were then crushed or left whole and covered with 0.5ml of digestion buffer. After overnight incubation, whole specimens were washed with ethanol and left to air dry. DNA from the digestion buffer was purified using two commercial kits: DNeasy Blood & Tissue Kit (Qiagen) or Qiaquick PCR Purification Kit (Qiagen) following manufacturer´s instructions. Three different variables within each protocol were tested: volume of digestion buffer, presence/absence of buffer ATL and insect integrity. A summary of the different tested methodological combinations can be found in Table S1.

Different pairs of primers were designed to amplify a variable region of the Cytochrome C Oxidase I gene (COI) from each species (Table S2). A 658bp region of this gene ("DNA barcoding" or "Folmer region" (Folmer et al., 1994; Hebert et al., 2003) has been widely used for invertebrate taxonomic identification. Sequences from the Folmer region from each species were aligned, and regions showing higher sequence diversity were targeted in primer design.

Initially, one set of primers for each species was used to amplify a region 120-155bp long. Where initial amplifications with the first pair of primers were successful, further PCR amplifications were performed using additional primers targeting overlapping regions of the COI gene (Table S2). This allowed the reconstruction of sequences of 201bp (positions 486-686) and/or 253bp (positions 394-646) in length for *M. domestica* (Table S2). All primers were designed using Primer3 (Untergasser et al., 2012) and Primer-BLAST (Ye et al., 2012) from reference sequences obtained from Genbank (Table S3).

Extracted DNA was amplified by PCR using the Qiagen Multiplex PCR Kit (1x Multiplex PCR Master Mix, 0.2µM of each primer and 5µl of DNA). Cycling conditions for a Prime Thermal Cycler were 15min denaturation at 95°C, followed by 40 cycles of 30s at 94°C, 90s at 55°C and 90s at 72°C and a 10min final extension at 72°C. Three PCR blanks were included in each PCR. PCRs for each extraction were repeated between 2-4 times until at minimum of two positive results were obtained with no evidence of contamination. Positive amplifications were checked in 1% agarose gels and purified using the GeneJET PCR Purification Kit (Thermo Fisher). Sanger sequencing was performed at the Durham University Sequencing Service (Durham, UK). Sequence electropherograms were aligned to the reference sequence using Mutation Surveyor (Softgenetics).

Consensus sequences were produced for each specimen that was successfully amplified and sequenced. These sequences represent the combined sequence information generated from each amplification for each specimen, and therefore required at least one sequence to have been produced for each region. Multiple sequences produced from different amplifications of the same specimen were compared, when available, to ensure each sequence generated from the same specimen were identical.

2.3. Statistical and population genetic analyses

The efficiency of each protocol and variable was evaluated measuring the number of positive PCR results produced. Any positive results thought to be due to contamination (i.e. if contamination was found in one of the extraction or PCR blanks) were not included. Results were evaluated using a Chi-squared (χ^2) test. Additional χ^2 tests were used to compare the contamination present with regard to the same variables. All statistical analyses were completed using IBM SPSS Statistics, Version 22.0 and graphs were produced using Microsoft Excel.

A genetic database of 294 published modern *Musca domestica* sequences from the studied COI gene region from various worldwide populations obtained from Genbank was compiled for comparative purposes (Table S3). These modern sequences were aligned using ClustalW Multiple Alignment (Thompson et al., 1994) and trimmed with BioEdit v 7.2.5. (Hall, 1999) to accommodate them to the sequenced positions in the ancient samples, after removal of external primers (positions 417-622 or 440-622).

For population analysis, sequences were grouped by country of origin. Groups with less than 5 individuals were not included in the analysis. Molecular diversity indices and population

pairwise F_{ST} values and their associated P values were calculated for all population pairs using the software Arlequin version 3.5 (Excoffier and Lischer, 2010) and 10000 permutations. As an additional measure of population affinity, the number and frequency of shared haplotypes between the ancient and modern populations were calculated with the same software.

To study further the relationship among the different haplotypes, Median Joining Networks (Bandelt et al., 1999) were calculated and drawn using Network and Network publisher version 2.1.2.5 (fluxus-engineering.com).

3. Results

3.1. Comparison of Protocols

Considering all amplifications, insect integrity had no significant effect on the ability to obtain DNA (χ^2=0.885, P value=0.347, df=1) (Figure 2, Table S4). While the Qiaquick kit produced more successful amplifications than the DNeasy kit, this is likely due to the inclusion of the buffer ATL in the latter, which was shown to significantly affect DNA amplification (χ^2=11.519, p value=0.001, df =1). However, DNA amplification success using only 0.1ml of digestion buffer with the Qiaquick kit was comparable to using 0.2ml of buffer, suggesting amplification was possible using smaller amounts of DNA extract.

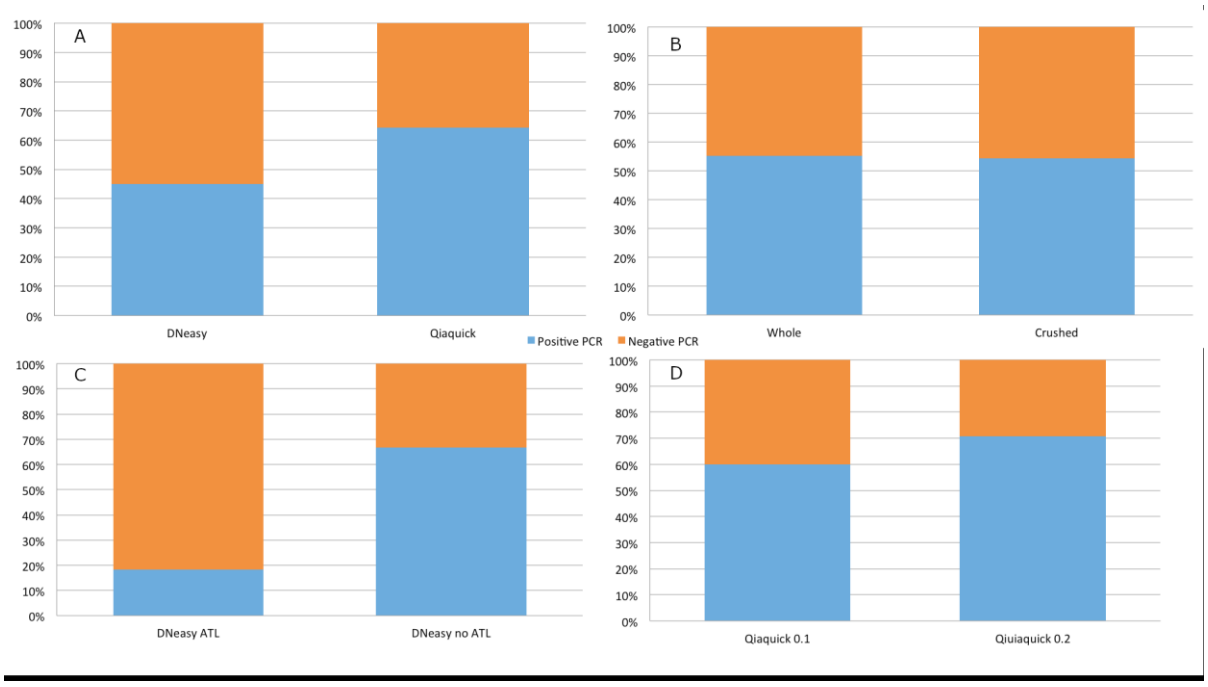

Figure 2. Comparison of the proportion of successful amplifications from all *M. domestica* fragments for different experimental variables. A) Extraction kit B) Insect integrity C) With/without ATL buffer added to the DNeasy kit (Qiagen) digestion buffer. D) With 0.1ml or 0.2ml of digestion buffer added when using the Qiaquick kit (Qiagen).

3.2. Ancient DNA of insect specimens from Qasr Ibrim

aDNA could be successfully amplified and sequenced in 9 out of 14 *Musca domestica* specimens using the primers MusCOIF1 and MusCOIRI, targeting a 99bp region of the Folmer's sequence (positions 548-646). No DNA could be amplified from *Trogoderma granarium* or *Sitophilus granarius* specimens using their respective primers. Of the nine *M. domestica* specimens that produced amplifiable DNA, PCRs were performed using two further sets of primers: MusCOIF2/MusCOIR2 and MusCOIF3/MusCOIR3, targeting a 82bp (positions 486-567) and 120bp (positions 394-513) fragments respectively, in order to expand the amplified region.

Positive amplifications were obtained in 9 specimens, with a sequence readable length (excluding primers) of 206bp (5 samples, positions 417-622), 183bp (3 samples, positions 440-622) and 113bp (1 sample, positions 509-622). Sequence alignments for the different amplifications and primer sets compared against a modern *M. domestica* sequence with accession number AY599508 can be seen in Table S5. PCRs were repeated for each specimen with the aim of producing multiple sequences that could be compared to check for errors, including contamination, and one consensus sequence was produced for each specimen (Table 3). However, it was not always possible to obtain multiple sequences from the same specimen, particularly when using primers MusCOIF3 and MusCOIR3. In the case of specimen CM2 a repeated PCR produced two different sequences, one with G to A mutations at positions 431 and 488 and another without (Table S5). This particular mutation is a common type of post-mortem DNA damage, a type 2 miscoding lesion (Gilbert et al., 2007a). As mutations at these positions were not observed in the first PCR for CM2, it was assumed that these base changes were due to the amplification of damaged fragments and did not represent sequence variation and were therefore excluded from the consensus sequence used in further analysis.

Compared to the reference sequence, cytosine to thymine base changes occurred at position 487 in all the sequences amplified with primers MusCOIF3 and MusCOIR3 with the exception of BM2. C to T base changes was also seen at position 616 in the sequences for specimens AM1 and BM2. Both base changes occurred in specimens CM2 and DM2. C to T changes are fairly common on aDNA sequences, and are also produced as a result of post-mortem DNA degradation due to Cytosine deamination (Gilbert et al., 2007a). However, in most cases the sequences generated by repeated PCRs for the same specimen consistently showed that these mutations were present. Amplification of a damaged DNA fragment is unlikely to produce identical sequences as deamination occurs at random, rather than at one specific position. The

similarity to other modern *Musca domestica* sequences, which are also polymorphic at these positions further suggests that this variation is not due to DNA degradation. Therefore, these C to T mutations can be considered to show endogenous genetic variation among the ancient *M. domestica* samples.

Specimen	Positions (excluding primers)	Haplotype (changes from reference sequence)	Matches in other populations
AM1	509-622	616T	-
BM1	440-622	487T	None
BM2	417-622	616T	10 (3.35%): 3 Egypt, 1 Saudi Arabia, 1 Israel, 1 India, 3 Japan, 1 US and Canada
BM3	440-622	487T	None
CM2	417-622	487T 616T	None
CM3	417-622	487T	None
DM1	417-622	487T	None
DM2	417-622	487T 616T	None
DM3	440-622	487T	None

Table 3. Consensus haplotypes obtained for the different *Musca domestica* specimens of Qasr Ibrim and geographic distribution of haplotypes in modern populations.

3.3. Comparison among population of *Musca domestica*

Table S6 shows the molecular diversity indices calculated separately for the 206bp (positions 417-622) and 183bp (positions 440-622) *Musca domestica* fragments. The number of haplotypes and polymorphic sites between both fragments is very similar, indicating that the use of the shortest fragment in population comparisons does not cause a substantial loss of resolution. Despite its small sample size, the population of Qasr Ibrim displays the highest haplotype diversity when the longest fragment is used, and is also among the five most diverse populations for the shortest.

Three different haplotypes (ht) were identified within Qasr Ibrim when fragments of 206bp (positions 417-622) or 183bp (positions 440-622) were considered: 616T(ht1), 487T 616T (ht2) and 487T (ht3) (Table 3). Haplotype distribution in Qasr Ibrim and other populations of the database is shown in Tables S7 (positions 417-622) and S8 (440-622). Only haplotype 616T from Qasr Ibrim, found in sample BM2, is shared with other populations in the database, with a total of 10 matches: 3 in modern Egypt, 1 in Saudi Arabia, 1 in Israel, 1 in India, 3 in Japan and 1 in US/Canada. While ht2 and ht3 are unique to the Qasr Ibrim group, mutations 487T and 616T defining these haplotypes are prevalent, and are found each in 4 other haplotypes in the database. In the modern populations 27 different haplotypes were identified at the 206bp fragment. From these, 20 were private (found only in populations from one location), and 19

singular (found in just one specimen). The most frequent haplotype among modern specimens (ht4) is however absent in the Qasr Ibrim sample.

To highlight further the relationship between the different haplotypes, a Median Joining Network analysis was conducted for the 206bp fragment. The analysis identified 26 variable positions, from which 19 had a single occurrence and 7 were hypervariable. An original analysis with default weight values, a transition:transversion ratio of 1 and an epsilon value of 0, showed multiple reticulations at hypervariable positions 433, 455, 496, 487, 562, 571 and 616 (Figure 3). Changing the transition:transversion ratio and increasing the epsilon value between 10 and 100 did not have any effect in resolving these cycles, so it was assumed that some of these positions might be homoplasic and had mutated more than once in the phylogeny. Therefore, a weight of 0 was given to hypervariable positions with 4 or 5 mutations: 562, 496, 487 and 616, and a weight of 5 to the ones with 2 or 3 mutations: 433, 455 and 571. The resulting Network contained only one reticulation involving the last three positions and involving haplotypes 5, 14 and 15 (Figure S1). Both Networks show a star-like phylogeny with a central node (ht4) surrounded mainly by haplotypes at one or two mutational steps (with the exception of haplotype 19). This pattern is compatible with a recent population expansion (Bandelt et al., 1995). Qasr Ibrim haplotypes are shared (ht1) or at one mutational step (ht2 and 3) from modern Egyptian haplotypes (ht1, ht20 and ht21).

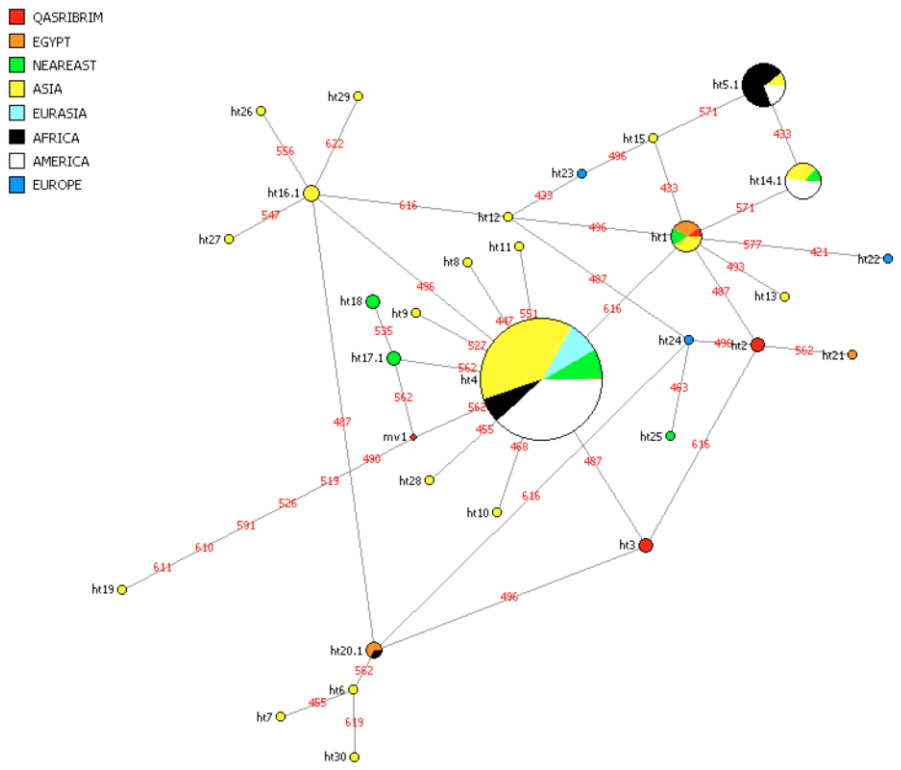

Figure 3. Median Joining Network analysis of population haplotypes. Default values were used (position weight: 10, transition/transversion ratio=1, epsilon=0).

F_{ST} genetic distances among populations were also calculated separately for positions 417-622 and 440-622. For both fragments Qasr Ibrim seems to be highly dissimilar from the other modern populations, with F_{ST} distance values ranging between 0.3 and 0.8. When the 183bp fragment is used, all the F_{ST} genetic distances between Qasr Ibrim and modern populations are significantly different with the only exception of modern Egypt, which also displays the lowest F_{ST} value (Tables 4 and S10). A similar situation is observed for the longer 206bp fragment, but in this case together with modern Egypt, Saudi Arabia and Japan also show low and non-significant F_{ST} values (Tables 4 and S9).

Populations	206bp fragment (positions 417-622)	183bp fragment (positions 440-622)
US/Canada	**0.78812**	**0.81018**
Japan	0.22523	**0.27214**
China	**0.75715**	**0.73511**
Thailand	**0.49836**	**0.54031**
South Korea	**0.52493**	**0.59298**
Saudi Arabia	0.33303	**0.39104**
Kenya	**0.52573**	**0.58057**
South Africa	**0.36343**	**0.43224**
Egypt	0	0.11277
Russia	**0.73988**	**0.70181**
Kazakhstan	**0.7561**	**0.71622**
Uruguay	**0.41286**	**0.47582**
Israel	**0.31689**	**0.35605**
Honduras	**0.4748**	**0.54286**
Chile	**0.56753**	**0.61239**
Indonesia	**0.24242**	**0.29627**
Zimbabwe	**0.52975**	**0.59257**

Table 4. Pairwise F_{ST} distance values between Qars Ibrim and modern population calculated for the 206bp and 183bp fragments. Significant values ($P<0.05$ are indicated in bold).

4. Discussion

4.1. Efficiency of protocols for DNA extraction from ancient insect material

The first part of this work assesses the efficiency of commercially available kits in extracting DNA from ancient insect samples. Positive results were obtained by PCR from samples using the DNeasy Blood and Tissue kit and the Qiaquick PCR Purification kit. While both kits can be used successfully to extract DNA, the present work shows that DNeasy Kit is more effective when ATL buffer is not used. Crushing the specimens has no impact on the success of the PCRs, meaning that it is possible to obtain DNA while preserving insect integrity, as

previously observed by Thomsen et al., 2009 in ancient and museum Coleoptera specimens. Our study therefore demonstrates that the same approach can be used efficiently in other insect groups with a thinner chitinous exoskeleton, such as Diptera.

The length of the amplified fragments has an impact on the overall PCR efficiency, as reflected in the less efficient amplification with primers MUSCOIF3 and MUSCOIR3, targeting a fragment of 120bp, when compared with the amplifications with the other two sets of primers, targeting fragments of 82 and 99bp. Considering this, we cannot discard the possibility of extracting endogenous aDNA from *T. granarium* and *S. granarius* by targeting shorter fragments of DNA using different sets of primers. DNA from *S. granarius* has been successfully amplified from waterlogged samples found in Roman Britain, when the fragments amplified were <100bp (King et al., 2009).

Another reason that could explain the lack of results from the grain pest species is the insect size. Although the housefly samples were relatively small, they were significantly larger than the grain pest specimens, and perhaps retained more amplifiable DNA. The adult housefly can grow up to 12mm (Skidmore, 1985) while *Trogoderma granarium* and *Sitophilus granarius* grow to a maximum length of 3.5mm (Peacock, 1993) and 5.0mm (Hoffmann, 1954) respectively. It may be possible to extract DNA from smaller insects if multiple specimens are placed in the digestion buffer at the same time. It is more likely that enough DNA will be released if an increased number of specimens are used, although if these specimens are genetically different, inconsistent results will be produced upon sequencing. This would obscure any potential contamination and prevent analysis at the intra-species level.

The success of the DNA amplifications reported here is noteworthy as these samples are significantly older than other insect specimens used in previous research (Strutzenberger et al., 2012; Virgilio et al., 2010). Additionally, the species examined here are smaller, with thinner chitinous exoskeletons than those involved in prior successful DNA extraction attempts. It was also possible for enough amplifiable DNA to be extracted with the Qiaquick kit using only 0.1ml of the available 0.5ml digestion buffer with no significant differences compared to extractions using 0.2ml of buffer. While the exceptional preservation of the specimens from this archaeological site may have contributed to the success of these extractions this study, along with evidence from past research, indicates that it is possible to amplify insect DNA from older assemblages using commercially available kits, without morphological damage to the specimen.

Contamination is a significant concern in any work involving aDNA. The fact that only modern populations from remote countries share haplotypes with the Qasr Ibrim samples, makes it unlikely for the DNA to be contaminated with DNA from modern local flies. Moreover, strict criteria for preventing contamination were used, including UV exposure of the specimens prior to DNA extraction, UV exposure of reagents and plastics used for extraction and amplification, use of extraction and PCR blanks and amplification of each extract a minimum of two times.

Based on the results presented here, we recommend the use of the digestion buffer described in (Gilbert et al., 2007b) followed by purification of DNA from the buffer using either the Qiaquick PCR purification kit or the DNAeasy kit without ATL to extract ancient DNA from desiccated insect specimens. Brief UV exposure of approximately 10 minutes per specimen removes surface contamination, and does not appear to affect the extraction of endogenous aDNA. To maximize aDNA recovery, primers should ideally be designed to amplify a region of less than 100 bp in length.

4.2. *Musca domestica*, genetic variability and biogeography

House-flies are one of the most frequent insects in settled areas and exploit a variety of environments, from herbivore dung to different types of garbage. In Egypt, as in other warm temperate environments, the species can produce a large number of generations over the year (Skidmore, 1985). They are common vectors in the spread of disease (cf. Greenberg, 1973; Panagiotakopulu, 2004; Skidmore, 1996) and are known to spread mechanically various diseases, for example, typhoid, cholera, yaws, tuberculosis and trachoma, the last an infection endemic in the Nile Valley causing blindness (Greenberg, 1973).

Only one 206bp haplotype (ht1), was shared between the Qasr Ibrim sample and modern *M. domestica* specimens from modern Egypt, Saudi Arabia, India, Israel, Japan and US/Canada. A similar pattern of population affinity emerges when considering F_{ST} genetic distances for this fragment, with the same populations except India, which was not included in the analysis, showing low non-significant F_{ST} values (Tables 4, S9 and S10).

The fact that 74% of the mtDNA COI haplotypes in the comparative database are population-specific suggests a highly differentiated genetic population structure with limited gene flow for *Musca domestica* populations. The same pattern has been also observed for single-strand conformation polymorphisms at the *16S2* and *COII* mitochondrial genes in the same species (Cummings and Krafsur, 2005; Krafsur et al, 2005; Marquez and Krafsur, 2002). Despite the ability of the housefly to travel both on the wing and by human agency, Marquez and Krafsur

399 (2002) have suggested that this lack of gene flow in modern populations could be due to
400 limited reproduction of houseflies within new environments.

401 The similarities observed between Qasr Ibrim and modern Egypt could be interpreted as
402 evidence of genetic continuity in the region over the centuries. In turn, the links observed
403 between Qasr Ibrim, Israel, India, Saudi Arabia, Japan and US/Canada together with the
404 observation of limited gene flow among modern populations could be suggestive of past
405 population movements between some of these areas. These could be associated with the
406 documented early trade in a variety of goods, including animals and plant products between the
407 Levant, the Arabian Peninsula and Roman Egypt (cf. Mclaughlin 2014; Tomber 2008;). Indian
408 spices were also traded, perhaps as early as 3000 BC, although the earliest records are debated.
409 Black pepper, with probable origins in south India, was recovered from the nostrils of the
410 mummy of Ramses II (1279-1213 BC) (Plu 1985; Sidebotham 2011) and the largest quantity
411 recovered was 7.5kg in a dolium (a type of ceramic storage vessel) from Roman Berenike, an
412 important trading port located on the Red Sea (Cappers, 2006). Cinnamon, also from India,
413 occurs at several sites on the Levantine coast dated to the 11^{th}- 10^{th} centuries BC (Cappers,
414 2006). Trade via the Red Sea with the Arabian Peninsula coastal sites appears to have begun in
415 the Predynastic period with imports of coral, urchins and a variety of sea shells (Mumford,
416 2012) and other materials, including obsidian (Khalidi, 2007, 2009), ebony and ivory from the
417 south (Cox, 2012; Trigger, 1987). This continued throughout the Roman and subsequent
418 periods up to and including the Islamic period (e.g. Van der Veen and Morales, 2017). In
419 addition, Lapis lazuli was imported from Afghanistan while the Near East provided silver and
420 resin (Garcia, 2017; Zarins, 1990, 1996). There was a significant increase in trading activities
421 during the Roman period with the use of the monsoon winds for sailing between India and
422 Arabia, perhaps reflected in the development of the Red Sea ports, Quseir, or in Greek *Myos*
423 *Hormos* (the Port of Mice), and Berenike (Cappers 2006; Van der Veen, 2011), which acted as
424 entry points for traded goods. These ports were critical to the movement of goods to and from
425 the Nile Valley (Facey, 2004). The establishment of cotton from the Indus valley in Arabia
426 during the Achaemenid period (c. 550-300 BC) (Bouchaud et al., 2018; Tengberg and
427 Lombard, 2002) and over to the Nile valley during the Roman period (Boivin and Fuller, 2009;
428 Bouchaud et al., ibid; Wild et al., 2007;) provides additional evidence for established long
429 distance links between these areas, although evidence from Qasr Ibrim also demonstrates a
430 possible African origin and a different domestication centre (Palmer et al., 2012).
431 Crops carried as on-board provisions or trading items across the Indian Ocean (Boivin et al.,
432 2009; Boivin and Fuller, 2009) would have aided the distribution of insect pests. The initial

introduction, however, of *M. domestica* and other pests in Egypt form part of a process initially linked with the spread of early agriculture from the Fertile Crescent, bringing new crops into Egypt (e.g. Allen, 1997; Fahmy, 2003; Fahmy et al., 2008). The spread of *M. domestica* from the Nile valley, probably in the dung of domestic herbivores (Skidmore 1985), is associated with the spread of agriculture across Europe (Panagiotakopulu and Buckland 2018). Although there is as yet limited fossil insect research from the Fertile Crescent and India, the house fly spread would follow similar pathways to the East and the West, an accidental transport with commodities, ballast and dunnage, etc., primarily in the dung of animals, which would also be part of the exchange in some cases. These links could potentially explain genetic similarities of the Roman Qasr Ibrim specimens with the modern populations of these regions. Whilst similarities with Japan may reflect eastward trade from India, those with USA/Canada could be a result of post-Columbian population dispersals associated with the introduction of new crops and animals in these areas and the burgeoning trade in food commodities, including livestock, across the globe.

To achieve a higher resolution, a diachronic DNA sequence database from these regions would be of paramount importance.

5. Conclusions

This study has confirmed that it is possible to extract, amplify and sequence DNA from desiccated ancient insects and provided ancient mtDNA results from *Musca domestica*. Although attempts to extract DNA from *Sitophilus granarius* and *Trogoderma granarium* were unsuccessful, the ability to obtain DNA from both species with minor modifications to primers should not be discounted.

Comparison with modern sequences revealed new genetic insights to the past movement of *M. domestica* populations. As the genetic sequences available for comparison were modern, it is not unexpected that some unique sequences emerge, although singular haplotypes are common even in modern populations. The sequences displayed here show a relatively high level of diversity, as three haplotypes were obtained from nine sequenced specimens. One of these haplotypes has been conserved in modern populations from Egypt, Saudi Arabia, Israel and India, all of which have long established routes of trade with Egypt, which go back to the Predynastic period. These links are important when it comes to the understanding of the biogeography of biological invasions from early synanthropic environments.

Further ancient insect DNA research will enhance our understanding of historical biogeography of modern cosmopolitan species, including the ones that are key for the spread of infectious

diseases.

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691 **Supplementary figure legend**

692

693 **Figure S1.** Median Joining Network analysis of population haplotypes. Modified values of
694 position weights were used as follows: 562, 496, 487 and 616 weight 0; 433, 455 and 571
695 weight 5, remaining weight 10. Transition/transversion ratio=1. Epsilon=0.